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13. SUPPLEMENTARY NOTES

14. ABSTRACT Increased solar radiation and other unknown factors induce excess melanin production in melanocytes accumulated in localized areas of the skin, leading to formation of benign moles and infrequently, malignant moles that progress to melanoma. My hypothesis in the current research project is that excess melanin production in melanocytes may cause physico-chemical constraints on the metabolic activities of DNA and RNA, which, in rare instances, may induce pro-survival responses, including mutations, in cellular DNA. This may lead to selective transcription and/or translation that support oncogenic transformation of these cells. To test this hypothesis, I induced long-term production of excess melanin in normal melanocytes using chronic tyrosine exposure and chronic UV radiation treatment. I then examined the cell biological changes induced by excess melanin production in these cells and found that excess melanin production caused a small increase in their proliferation. More interestingly, chronic melanin induction in melanocytes derived from fair-skinned individuals, but not dark-skinned individuals increased the migration and soft-agar colony formation ability of the melanocytes, suggesting that melanin or melanin biosynthetic machinery may play a role in oncogenic transformation. Gene expression profiling of low melanin and high melanin containing normal melanocytes revealed distinct expression patterns associated with different signaling networks. Functional studies are currently underway to determine if knock-down of tyrosinase activity, which decreases melanin content in cells, abrogates the transformative potential of chronic melanin induction.

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Melanin, melanocyte, melanoma, oncogenesis

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Introduction

UV radiation is a major occupational risk factor of melanoma in the U.S. combat soldiers. Melanin pigment is the primary defense in the skin and eyes against the mutagenic effects of UV radiation. Through poorly understood mechanisms, melanin forms an envelope over the nucleus, and prevents UV-radiation damage to nuclear DNA. However, many melanoma tumors originate from melanocytes within highly pigmented nevi. In such cases, UV-damaged melanin is thought to produce mutagenic reactive oxygen species[1], which may contribute to oncogenesis. However, UV-radiation damage is not present in melanomas that develop from nevi in non-sun-exposed areas of the body like mucous membranes or the soles of feet. In these instances, it is compelling to question the role of undamaged melanin in oncogenesis. To answer this question, it is imperative to separate the UV-protective role of melanin from its other cellular activities, and determine the effects of excess melanin in nevi melanocytes from a conceptually different perspective. The current study is focused on exploring the oncogenic potential of excess melanin production in melanocytes in the presence and absence of UV damage. This study may have a potential impact on novel therapeutic development and a direct impact on the formulation of melanoma risk prevention and chemo-prevention strategies for combat soldiers in the U.S. army.

Melanin is synthesized in melanocytes and transferred to other cell types like keratinocytes to provide a homogeneous pigmentation in the skin. The enzyme tyrosinase is the rate limiting factor in the enzymatic conversion of the amino acid tyrosine into melanin. Melanin is stored in melanosomes in the cytoplasm and is also present in the nucleus, although its nuclear functions are unknown. Melanin itself seems to promote cellular differentiation, which may be a contextual role, since this role seems to be promoted by the oncogenic MITF transcription factor[2,3]. It has been suggested that in its UV-protective role, melanin forms an envelope over the nucleus and DNA. This mechanism may also protect DNA from genotoxic/cytotoxic drugs, either through direct sequestration of the drugs or by acting as a physical barrier that prevents access to the DNA[4,5]. Similarly, it is possible that excess melanin in cells that constitute benign nevi may cause physico-chemical constraints on DNA and RNA metabolic activities, such as transcription and translation. In rare instances, this may induce pro-survival responses, including mutations, in cellular DNA. This may lead to selective transcription and/or translation that support oncogenic transformation of these cells. **I hypothesized that excess melanin production may induce a pro-survival response in cells, leading to selective transcriptional regulation that su pports oncogenic transformation of mel anocytes.** To test this hypothesis, I proposed the following two specific aims.

Aim 1- Determine transcriptional changes in melanocytes after inducing excess melanin production.

Aim 2- Determine the transformation potential of excess melanin production in melanocytes.

Body

The experiments in the above two specific aims have been split into separate tasks and the progress of the experiments in these tasks is described below.

Task1A: <u>Induce melanin production in melanocytes and perform UV-radiation treatments.</u>

Two sets of low melanin containing primary neonatal melanocytes (LP1 and LP2) were obtained from two different sources- Lonza (Walkersville, MD) and Invitrogen (Carlsbad, CA). Two sets of high melanin containing primary neonatal melanocytes (HP1 and HP2) were obtained from Invitrogen (Carlsbad, CA). These sets represent melanocyte cells from two different ethnic backgrounds. Primary adult melanocytes from Lonza and ATCC (Manassas, VA) were also obtained. However, the adult melanocytes were overly sensitive to the treatments in this study, and were not pursued further. All primary melanocytes in this

study were grown in 254 media with HMGS-2 serum supplement in 10 cm dishes. Long-term chemical induction of melanin synthesis in these cells was performed by inclusion of 0.5 mM of L-tyrosine in the medium. At this dose, L-tyrosine induced high levels of pigmentation in these cells, without affecting their short-term growth. (Figure 1A). UV-radiation induction of melanin synthesis was performed as described[6] by daily treatments of the cells using an FS-40 sunlamp (National Biological, Twinsburg, OH). This lamp emits a continuous spectrum of UV radiation from 270 to 390 nm, with a peak emission at 313 nm; approximately 65% of the radiation is within the UVB (290-320 nm) range. The UVB output of this bulb averaged 1.35 $J/m^2/sec$, at a tube to target distance of 23 cm as measured with an IL-1700 radiometer (International Light, Newburyport, MA). Approximately 1×10^6 melanocytes were plated in 10 cm tissue culture dishes, the medium was removed and the cells were re-suspended in PBS. The cells were exposed to 50 J/m^2 dose of UVB radiation. The PBS was removed and the cells were then re-suspended in tissue culture medium. Different non-erythema doses of UVB were tested on the cells initially. The dose of 50 J/m^2 induced the highest levels of melanin over long periods of time, with no apparent toxicity to the cells (Figure 1B). For quantifying melanin, cell pellets from 10^6 cells/sample were collected by centrifugation. The cell pellets were suspended in 1 ml of 1 N NaOH solution and incubated at 80^0 C for 2 h. The resulting cell lysates were centrifuged at 6000 rpm for 5 min. The pellet containing DNA and other cell debris was discarded, the optical absorbance of the clarified lysates at 470nm wavelength was measured, and melanin content was quantified against a standard curve generated using synthetic melanin (Sigma, St. Louis, MO). Long-term melanin induction treatments were carried out for a period of 6 months. For the long-term chemical induction, tyrosine containing media was replenished every 3 days, while the UV-radiation treatments were performed every day. The daily UV-radiation treatments for 6 months emulated the occupational exposure of active military personnel in tropical regions. Melanin levels were quantified every 15 days. After completion of the incubations, one set of plates was used for cell lysate preparation in RIPA lysis buffer and stored in -80 ^{0}C freezer for future protein analysis. A second set was used for RNA isolation. Task 1A has been successfully accomplished.

Task 1B: Gene expression profiling to detect melanin-induced transcript changes.

Total cellular RNAs from primary melanocytes in the above experiments were purified using the RNeasy kit (Qiagen, Valencia, CA). RNA quality control (RNA Integrity Number determination) was performed on an Agilent Analyzer. Whole genome expression profiling was performed at the University of Texas-Houston, microarray core facility using the Illumina human HT12 beadchip microarray, which contains over 48,000 unique probes representing 25,000 annotated genes, gene candidates and splice variants per array (derived from the RefSeq and UniGene databases) was used for this experiment. Initial quality analysis, probe annotation and normalization were carried out by the core facility. Further analysis of the data was performed by PI using Ingenuity Pathway Analysis (IPA), Cluster and Treeview programs. IPA signaling network analysis showed that p53 signaling network was the most significantly dysregulated by long-term UV-radiation treatment of low pigmented melanocytes, LP1 and LP2 (Figure 2A). The p53 regulatory components like p21/Waf1 were significantly upregulated in these cells. Other major signaling networks affected in the LP cells upon melanin induction were cell cycle regulatory pathways and cancer tissue response factors (Figure 2A). In the high pigmented melanocytes (HP1 and HP2), oxidative phosphorylation, which is the final phase of aerobic respiration, was most dysregulated (Figure 2B). This dysregulation involved downregulation of expression of the whole program of oxidative phosphorylation genes. This data is extremely surprising, since cellular metabolic regulation by long-term melanin induction has not been observed before. This will be explored further in detail in the coming months. Other major dysregulated networks in the melanin-induced HP cells were protein ubiquitination pathways and mitotic regulation of Polo like Kinases (Figure 2B). Interestingly, expression of the melanin synthetic machinery like the POMC gene, which translates into αMSH, βMSH, and γMSH peptides, tyrosinase

(TYR) and tyrosinase related protein (TYRP1) genes was slightly downregulated by the long-term melanin induction (Figure 2C). Expression of micropthalmia associated transcription factor (MITF) and dopachrome tautomerase (DCT) was downregulated, while the expression of the melanocortin 1 receptor gene (MC1R) was upregulated in the LP cells and to a greater extent in the HP cells (Figure 2C). To determine if these observed changes are a result of melanin induction or cellular responses to UV radiation treatment, long-term chemical induction of melanin by tyrosine treatment was also performed. However, gene expression analysis for this experimental set has not been performed yet due to the lack of the requisite controls for this experiment. Since tyrosine is an essential amino acid, chronic availability of high levels of tyrosine may induce metabolic changes in cells, independently of melanin. To separate this potential effect of tyrosine from the effect of increased melanin on the cells, it was necessary to generate cell clones lacking the ability to make melanin, and then treat them with tyrosine. These cells will be appropriate controls for this experiment. To do this, I had proposed to use a retroviral transduction system to make stable shRNA expressing clones of primary melanocytes. However, this part was significantly delayed due to technical difficulties (described in Task 1C), and this is the reason for my EWOF request.

Task 1C: <u>Validation of relevant gene expression changes identified by the gene expression profiling.</u>
To validate the results obtained in the above microarray experiment and to generate primary melanocyte controls for chemical induction of melanin (as described in Task 1B), I proposed shRNA-based stable knockdown of melanin production in the cells. To determine the best target enzyme for this purpose, I first performed transient siRNA knockdown of enzymes specifically involved in melanin production- phenylalanine hydroxylase, tyrosinase, tyrosine hydroxylase and dopachrome tautomerase. Among these, tyrosinase exhibited the best reduction in melanin levels in the primary melanocytes, as well as melanotic melanoma cells (Figure 3A). I then used the pSilencer 5 Retro System (Ambion, Austin, TX) for generating tyrosinase shRNA vectors. However, this retroviral system could not induce stable knockdown of tyrosinase in the primary melanocytes, although it did so in melanoma cells. The relatively slow cell division rate of primary melanocytes, apart from other unique characteristics may have prevented efficient retroviral delivery and shRNA expression in these cells. After multiple failed attempts, I used the pGIPZ lentiviral system from Open Biosystems, Huntsville, AL, (Figure 3B) to generate tyrosinase shRNA containing virus. I have only recently been able to successfully transduce the primary melanocytes with this vector and efficiently knock-down melanin production (Figure 3C). This however has significantly delayed the timeline for the progress of this research. Hence, I am requesting the EWOF, which will enable me to complete this research in its entirety. I am currently performing long-term tyrosine treatment of these cells. After these treatments, I will perform gene expression microarray analysis on these cells as well as the melanocytes with chemically induced melanin (from Task1B).

<u>**Task 2A : Determination of the transformation potential of melanin producing cells, +/- UV-stress**</u>
<u>Changes in Cell Cycle:</u> The chemically induced and UV-radiation-induced, excess melanin producing melanocytes from Task 1A were tested for changes in cell cycle profiles, using a standard Propidium Iodide-based, flow cytometry assay. No noticeable changes in cell cycle profiles were observed between the melanin-induced and uninduced cells (not shown).
<u>Changes in Proliferation:</u> Cell proliferation over a period of one week was measured using the CellTiter-Blue cell proliferation assay (Promega, Madison, WI). A small increase in proliferation was observed in the LP cells with melanin induced by tyrosine treatment and UV-radiation-treatment, but not in the HP cells (Figure 4A).
<u>Cellular Transformation Assays:</u> To determine transformation potential of the excess melanin producing cells, a scratch assay was performed on the LP cells growing in 10 cm dishes, and the migration of cells into a cleared area was photographically documented. The cells induced to synthesize melanin showed a

higher rate of migration over a period of 2 weeks. Migration of the tyrosine treated cells was slightly reduced compared to the UV-radiation treated cells (Figure 4B). Foci formation and anchorage independent growth of the differentially treated melanocytes was determined using soft agar growth assay. Melanin induction by tyrosine treatment of primary melanocytes slightly increased foci formation by the LP cells and increased the size of the foci in the UV treated cells (Figure 4C). This suggests oncogenic transformative potential of melanin induction in the melanocytes. This study will be repeated with melanocytes and melanoma cells in which melanin synthesis was stably knocked down with shRNA against tyrosinase (Task 2B). Although the newly transformed cells may not have the ability to metastasize, the metastastic potential of the transformed melanocytes will nevertheless be tested using a modified Boyden Chamber Assay (BD Biosciences, San Jose, CA) in the next few months.

Task 2B: Validation of the transformation potential

The same assays described in Task 2A will be carried out with the tyrosinase shRNA knockdown cells that have been described in Task1C. This experiment is necessary to eliminate the effects of L-tyrosine on cellular homeostasis, which may be independent of melanin induction. However this task has been delayed due to delay in Task 1C.

Task 2C: Manuscript preparation and submission and meeting presentation

The data generated in this work will be written into a manuscript for publication in an appropriate journal. A positive correlation of the role of melanin in oncogenic transformation will be a completely new discovery and will have the potential to be published in a very high impact journal like Nature or Science. A poor/negative correlation or indirect association will also be an important finding because this unique investigation will reveal many new clues for understanding melanocyte and melanoma biology. Such a finding will be the basis for a good publication in a high to medium impact journal like the Journal of Investigative Dermatology.

Key Research Accomplishments

- This research until now has yielded data which suggest that excess melanin production induces unique gene expression changes in the lightly pigmented versus heavily pigmented melanocytes.

- While upregulation of the p53 signaling pathway was an expected effect of UV-radiation treatments, downregulation of oxidative phosphorylation in the highly pigmented cells was unexpected. While it is too early to make any conclusions based on this data, it can be hypothesized that downregulation of oxidative phosphorylation may reduce the levels of respiration in the cells, leading to lower levels of free radical generation. This effect may be mediated by melanin and may be a protective mechanism that prevents free-radical damage to melanin. Specific genes identified by the microarray analysis, eg. SOD1, CYP1B1 and CYCS will be explored further.

- Most interesting is the fact that except the gene encoding MC1R receptor, none of the genes involved in the melanin biosynthetic pathway were upregulated by the long-term production of excess melanin. In fact, MITF and DCT genes were potently downregulated, and the rate limiting, melanin synthetic enzyme Tyrosinase, was slightly downregulated. This suggests that MC1R may be critically regulated by melanogenic stimuli like UV radiation or other environmental factors. MC1R in turn may induce post-translational activation or stability of the melanin biosynthetic machinery to increase melanin production in melanocytes. Also, the levels of upregulation of MC1R were different between the LP and HP melanocytes, suggesting that melanogenic stimuli

may not similarly activate MC1R in the melanocytes of individuals from two different ethnic backgrounds. Many Caucasian populations with low melanogenic responses have sequence variations in the MC1R gene that reduce its activity[7]. A similar variation in the LP melanocytes may be responsible for lower melanogenic responses in these cells.

- Long term production of excess melanin increases the proliferation and transformative potential of low pigmented primary melanocytes as suggested by the soft-agar foci formation assay. However, some of the important validation experiments with Tyrosinase gene inactivation are still pending, and completion of these experiments will yield valuable information regarding the role of melanin in melanocyte transformation and oncogenesis.

Reportable Outcomes

Many research studies have reported the oncogenic effects of UV-radiation and the protective role of melanin on melanocyte homeostasis and oncogenesis, However, the role of long-term induction of excess melanin, irrespective of UV-damage, in prevention or promotion of melanoma has not been studied. This is especially intriguing in the instances of melanomas that arise in areas of the body with no UV-damage, but start as highly pigmented nevi. Whether high melanin accumulation in such malignant nevi may be a precursor to oncogenesis has not been determined. This research is the first to explore this question. Initial cell biological and gene expression observations suggest that long-term melanin induction may increase the transformation of melanocytes with low basal melanin levels, compared to cells with high basal melanin levels. If this is supported by the functional studies that will be performed in the next 6 months, it will be an extremely important outcome that will be published in a peer reviewed journal. In addition, I would like to present this research at a national or international skin cancer meeting.

Conclusions

Long-term UVB radiation treatment induces unique gene expression changes in melanocytes derived from lightly pigmented individuals (LP cells) compared to melanoctyes derived from dark pigmented individuals (HP cells). Additionally, cell proliferation, migration and foci formation assays showed that long-term melanin induction by tyrosine treatment or UV radiation increased the growth and transformative potential of the LP cells, but not the HP cells. Whether these changes are mediated by chronic production of melanin, or damage caused by UVB radiation will be determined in the coming months, using cells with stable knockdown of tyrosinase activity.

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Figures

Figure 1A

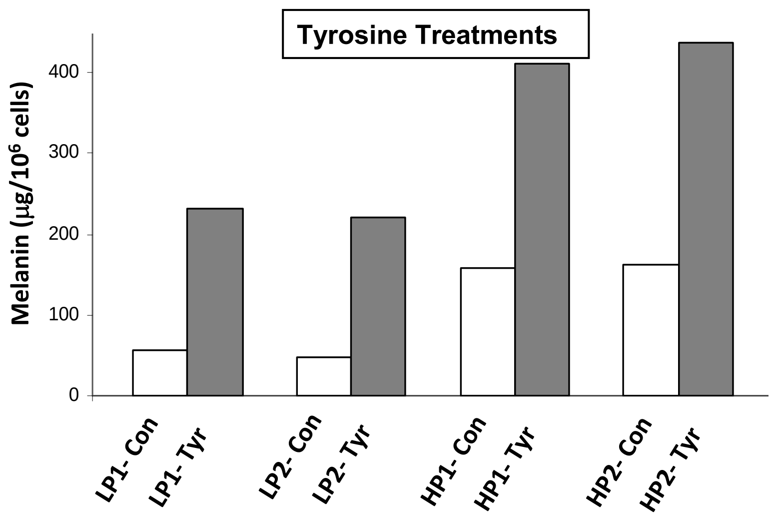

Figure 1B

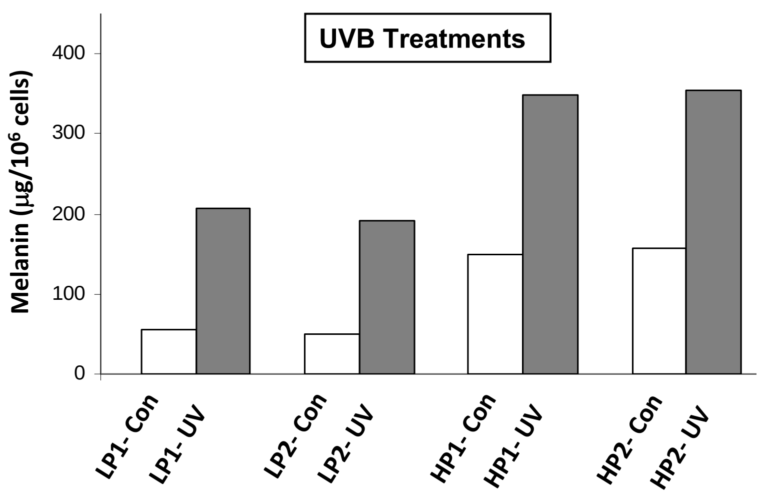

Figure 1: ***(A & B)*** The low pigmented melanocytes (LP1 and LP2) and the high pigmented melanocytes (HP1 and HP2) were treated with tyrosine or UVB radiation for a period of 6 months. For the tyrosine treatments, the cells were replenished with 0.5M tyrosine (Tyr) containing media every 3 days, and for the UV radiation treatments, the cells were treated with 50J/m^2 UVB radiation (UV) every day, as described in Task1 A. Control (Con) cells were grown alongside the treated cells and underwent similar changes in media, without tyrosine or UVB treatments. Melanin levels at the end of the treatment period was spectrophotometrically quantified. The tyrosine treatment induced higher levels of melanin in the cells compared to UV radiation. HP cells showed a higher accumulation of melanin compared to the LP cells. Data is representative of triplicate samples. x-axis, cells and treatments. Y-axis, concentration of melanin in micrograms per million cells.

Figure 2A

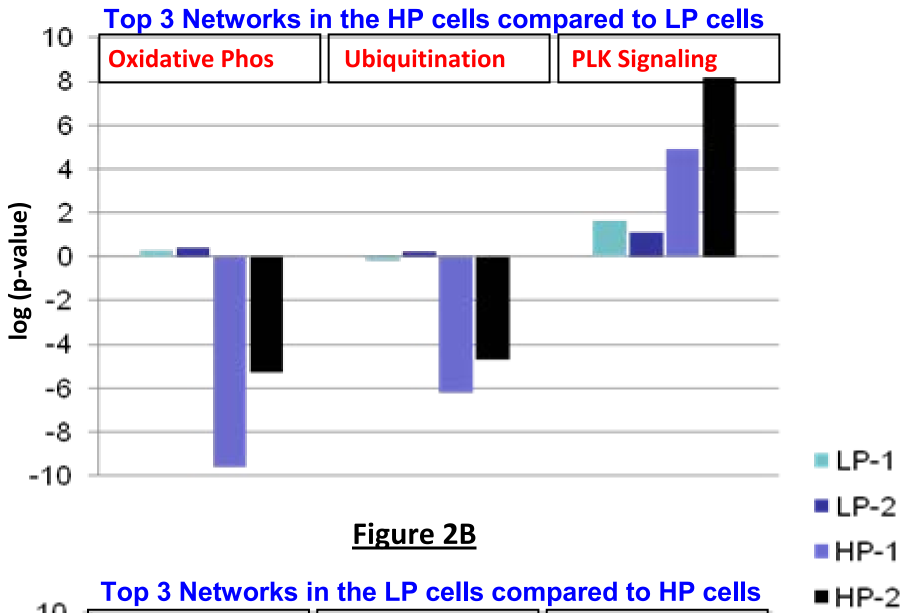

Figure 2B

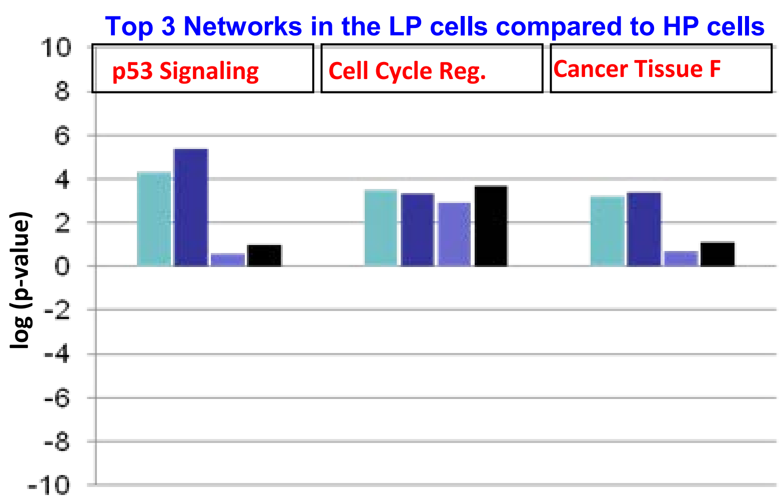

Figure 2C

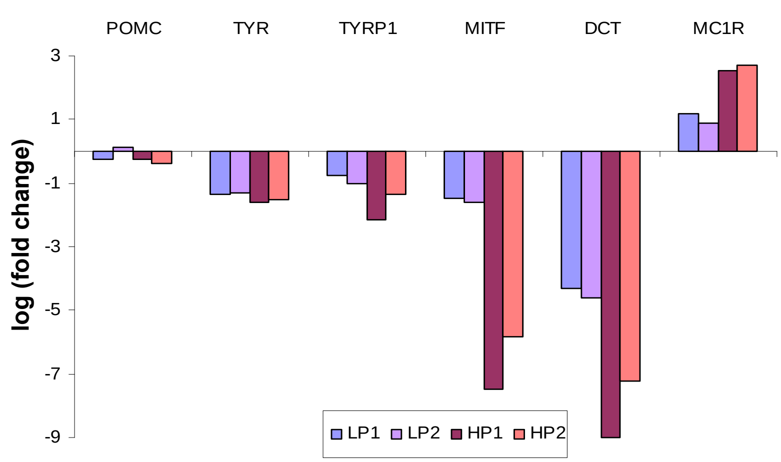

Figure 2: (***A & B)*** After 6 months of daily UVB radiation treatments, the cells were incubated for 72 h and total RNA was extracted from the UV-treated and control cells, and a whole genome gene expression analysis was performed using Illumina HT12 bead-chips. The data was normalized, log transformed and a gene expression network analysis was performed using Ingenuity Pathway Analysis (IPA). The data showed that Oxidative phosphorylation was the most dysregulated network in the UV-treated HP cells compared to the LP cells, while p53 signaling was the most dysregulated network in the LP cells, compared to the HP cells. Interestingly all the oxidative phosphorylation genes in the HP cells were downregulated, while the p53 signaling genes were upregulated in the LP cells.
(*C*) Long-term UV radiation caused a decrease in the expression of the tyrosine biosynthetic enzymes, with the exception of MC1R in the LP and HP cells. Some of these changes were more pronounced in the HP cells. Data represents log fold change ratio of treated cells versus control cells.

Figure 3A

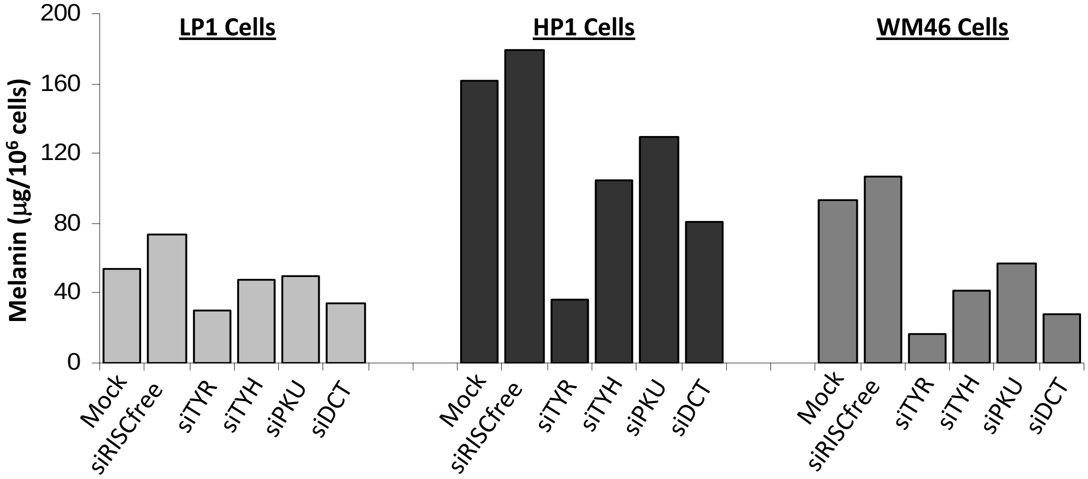

Figure 3B

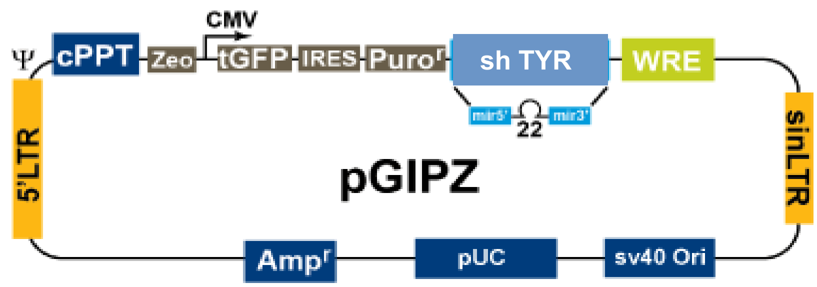

Figure 3C

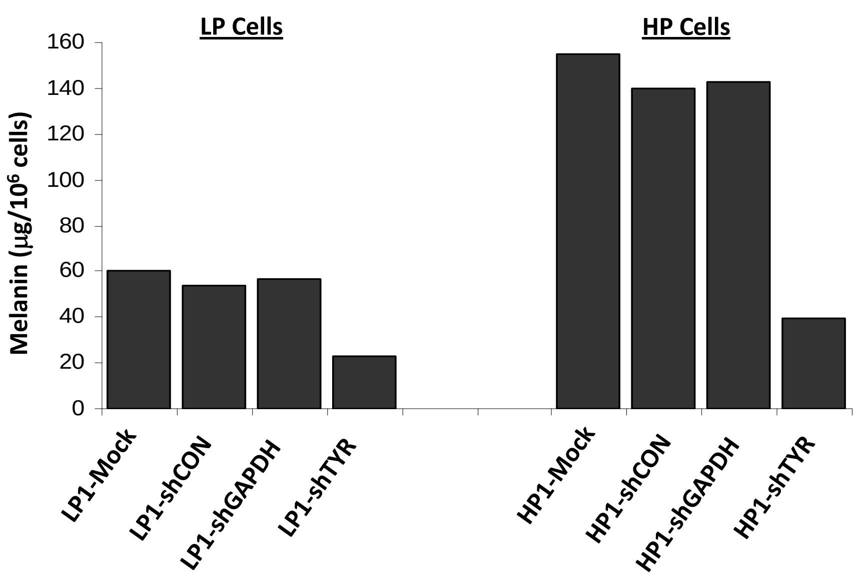

Figure 3: (A) The LP1, HP1 and WM46 melanotic melanoma cells were transfected with siRNAs directed against melanin biosynthetic enzymes, tyrosinase (siTYR), tyrosine hydroxylase (TYH), phenlyalanine hydroxylase (siPKU), and dopachrome tautomerase (siDCT). The cells were incubated for 96 h and harvested for melanin quantitation. The siRISCfree siRNA was used as a control, which slightly increased melanin levels in the cells. All siRNAs directed against the melanin biosynthetic enyzmes reduced the amount of melanin in varying amounts. The tyrosinase siRNA was most Effective, and consistently reduced melanin levels in all cells. ***(B)*** Tyrosinase shRNA sequence was inserted into the pGIPZ lentiviral vector containing a CMV polymerase II promoter and a WRE sequence for efficient shRNA processing. ***(C)*** The LP1 and HP1 cells were transduced with virus containing empty vector (shCON), GAPDH (shGAPDH) or tyrosinase (shTYR) shRNAs. One month after transduction, puromycin selected cells were quantified for melanin. The data shows efficient (80%) knockdown of melanin synthesis in the HP cells and about 70% knockdown in the LP cells.

Figure 4A

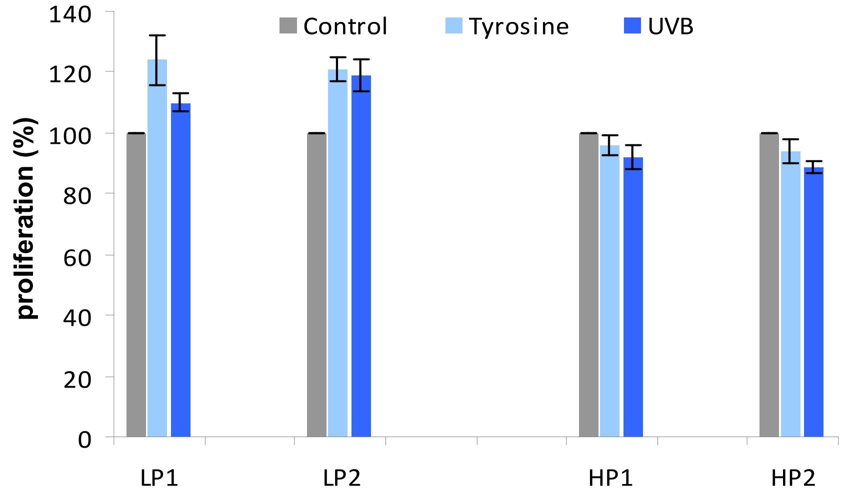

Figure 4B

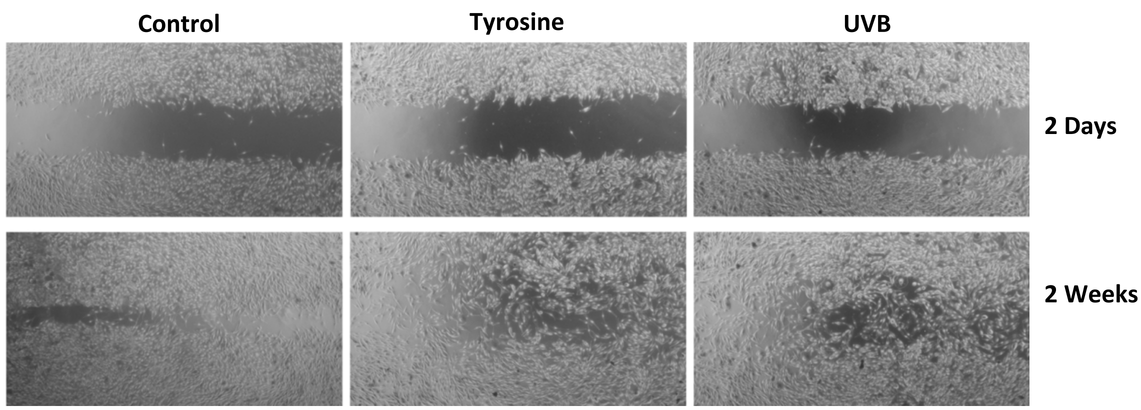

Figure 4C

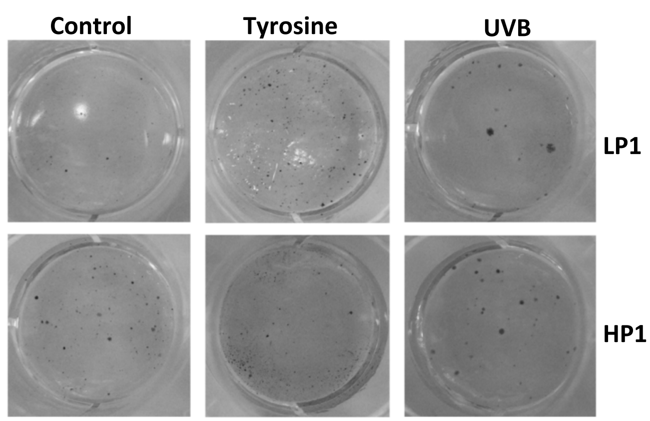

Figure 4: ***(A)*** After 6 months of tyrosine or UV-radiation treatments, the LP and HP cells were seeded in 96-well plates at a density of 1000 cells/well and incubated for 7 days, after which the CellTiter-Blue proliferation assay was performed. The data showed that tyrosine and UV treatments increased the proliferation of the LP cells, but not the HP cells. ***(B)*** A cell migration "scratch" assay with the LP cells showed that the 6 month tyrosine and UV treatments slightly increased the migration rate of the cells. ***(C)*** A soft agar colony formation assay showed that the untreated LP cells formed fewer colonies compared to the HP cells, with a mean diameter of 0.5 mm per colony. Tyrosine treatment increased the number of colonies, but not the mean size of the colonies in the LP cells. In the HP cells, there was no significant change in the number or size of the colonies in the tyrosine treated cells. UV treatment however increased the mean size of the colonies in the LP cells to 3 mm, but only about 1 mm in the HP cells.